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PRINCIPAL INVESTIGATOR: Stephen D. Hursting, Ph.D.

CONTRACTING ORGANIZATION: University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

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<p>Wnt-1 transgenic (TG) mice develop mammary tumors at a high rate by 1 year of age due to mammary gland overexpression of the Wnt-1 oncogene. We have shown that p53-deficiency accelerates mammary tumorigenesis in these mice, with 100% of p53-/- Wnt-1 TG mice and p53+/- Wnt-1 TG mice dead from mammary tumors by 5 months and 9 months of age, respectively. To test their response to cancer preventive regimens, we randomized 104 female p53+/- Wnt-1 TG mice (5 weeks of age, 20 mice/treatment) to receive: 1) control diet (AIN-76A diet); 2) fenretinide (AIN 76A diet with 0.04% w/w fenretinide); 3) fluasterone (AIN-76A diet with 0.2% fluasterone); 4) soy (AIN-76A diet with 0.45% phytochemical-enriched soy extract); or 5) a calorie restriction regimen (40% reduction in carbohydrate calorie intake relative to the control group). All mice were euthanized once a tumor reached 1.5 cm in diameter. We found that, relative to the control group (MTD=15.7 weeks), the fenretinide (MTD=23.1 weeks, p=0.01) and fluasterone (MTD=23.1 weeks, p=0.006) and soy (MTD =21 weeks, p=0.04) groups displayed moderate but significant delays in tumor development and death, while the calorie restricted group (MTD > 40 weeks; p<0.001) experienced highly significant delays in spontaneous mammary tumor development. Similar trends were observed with these agents in male and female p53-/- Wnt-1 TG mice and female p53+/+ Wnt-1 TG mice; however, the former genotype appears to develop mammary tumors too rapidly to be an effective model while the latter genotype is too slow and variable relative to the p53+/- Wnt-1 TG mice. We conclude that p53+/- Wnt-1 TG mice provide a rapid and sensitive model of spontaneous mammary tumorigenesis for characterizing breast cancer prevention strategies.</p>					
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Stephen J. Hustring
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Annual Report: Year 2

Characterization of Wnt-1 Transgenic Mice (With and Without p53-Deficiency) as Models of Spontaneous Mammary Tumorigenesis for Chemoprevention Studies

Principal Investigator: Stephen D. Hursting, Ph.D., M.P.H.

Institution: M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, TX 77030

Phone: (713) 745-2492

1.0 INTRODUCTION

1.1 Cancer Prevention Studies in Transgenic Mice

The recent development of mouse strains with overexpressed or inactivated cancer-related genes is providing highly sensitive and relevant models for studying the carcinogenesis process and ways to interfere with that process (1). We have previously capitalized on the susceptibility of p53-deficient mice to spontaneous tumor development to characterize interventions that can offset the loss of one or both alleles of the p53 tumor suppressor gene (2-10). The majority of p53-deficient mice develop hematopoietic neoplasias or sarcomas at an early age; spontaneous mammary tumors are rare in these mice.

We have previously shown that several nutritional and chemopreventive interventions delay the onset of spontaneous tumorigenesis in male and female p53^{-/-} mice (2-10). Since the proposal was submitted, we have reported that calorie restriction (CR), a well-documented and potent modulator of rodent tumor development, including carcinogen-induced mammary tumors, significantly delayed spontaneous tumorigenesis and slowed lymphocyte cell cycle traverse in male p53^{-/-} and wild-type (p53^{+/+}) mice (6). The tumor-delaying effect of CR was virtually identical in the two genotypes, even though tumor development was much faster in p53^{-/-} than p53^{+/+} mice, indicating that the mechanism underlying the anti-tumor effect of CR is p53-independent.

We also reported (3) that dehydroepiandrosterone (DHEA), a steroid with anti-inflammatory and cancer chemopreventive activity in several tumor models, including chemically induced mammary tumor models (11), delayed spontaneous tumorigenesis by over 80% and specifically suppressed lymphoma development in male $p53^{-/-}$ mice. Also, the DHEA analogue 16- α -fluoro-5-androsten-17-one, which may be a more promising chemopreventive agent given its lack of androgenic and estrogenic activity and appetite suppression relative to DHEA, also suppresses spontaneous lymphoma development (4). In addition, we found that DHEA and its analogue increased thymic expression of the $p53$ -dependent cell cycle regulator *Waf-1/p21* and decreased thymic expression of the $p53$ -related apoptotic regulator *Bcl-2* (with no effect on *Bax*, a negative inhibitor of *Bcl-2*) in both $p53$ -knockout and wild-type mice (5). We also found that these chemopreventive steroids in our hands do not decrease nucleotide pools (their purported mode of action; 11) but do decrease nitric oxide generation and down regulate the expression of the inducible nitric oxide synthetase gene (8).

We have not yet evaluated the effect of 4-HPR on spontaneous tumorigenesis in $p53^{-/-}$ mice, although we have evaluated its effects in a p-cresidine-induced bladder model in $p53^{+/-}$ mice (manuscript in preparation) and in an in vitro prostate cancer system (12). In addition, preliminary data in male $p53^{-/-}$ mice in our laboratory suggest that 4-HPR increases thymic expression of *Waf-1/p21* and decreases the expression of *Bcl-2* but has no effect on *Bax* expression. These changes in gene expression are consistent with those observed in response to DHEA and other efficacious interventions in our laboratory. 4-HPR has been reported to increase expression of retinoic acid receptor (RAR)- β in normal mammary epithelial cells, but not tumor cells (13). Collaborator R. Lotan and colleagues have shown increased RAR- β to be associated with the chemopreventive efficacy of retinoids in head and neck cancers (14) and also in the response of nitrosomethylurea-induced mammary tumors in Fischer rats to 4-HPR (personal communication).

$p53$ -Deficient Wnt-1 Transgenic Mice

Future progress in the nutritional modulation and chemoprevention of breast cancer may be facilitated by the use of animals with specific genetic susceptibilities for spontaneous mammary

tumorigenesis. This may overcome some of the limitations of existing mammary carcinogen models, which are characterized by high dose, acute exposures of a genotoxic carcinogen generally not encountered by humans. *Wnt-1* transgenic (*Wnt-1 TG*) mice are predisposed to the development of mammary adenocarcinomas. *Wnt-1* is a mouse proto-oncogene which encodes a cysteine-rich glycosylated secretory protein normally expressed during mouse embryonic development and not expressed in the normal mammary gland (15). *Wnt-1 TG* mice display hyperproliferative mammary glands and high rates of spontaneous mammary adenocarcinomas within the first year of life due to the ectopic expression of the *Wnt-1* oncogene in the mammary gland under the influence of the mouse mammary tumor virus promoter (16).

The generation of bi-transgenic mice by crossing p53-deficient mice to *Wnt-1 TG* mice results in a spontaneous mammary tumor model in which 100% of the mice develop type B adenocarcinomas, with the rapidity of tumor development dependent on p53 status. The median time to a 1.5 cm mammary tumor (our criteria for euthanasia) in p53^{-/-}:*Wnt-1 TG* mice is ~4 months of age (~10 weeks on study) compared to ~7 and 10 months for p53^{+/-}:*Wnt-1 TG* and p53^{+/+}:*Wnt-1 TG* mice, respectively (Table 1). In addition, mammary tumors from *Wnt-1 TG* mice with wild-type p53 tend to be more organized, fibrotic, and differentiated relative to tumors from p53-deficient *Wnt-1 TG* mice (17). **The responsiveness of either the *Wnt-1 TG* mice or the p53-deficient *Wnt-1 TG* mice to chemopreventive agents or other tumor modulating regimens has not previously been studied.**

Spontaneous mammary carcinogenesis in these mice involves alterations in two molecular pathways known to be involved in human mammary tumor development. Thus, the specific genes along those pathways provide critical targets for evaluating the mechanism underlying any observed modulating effects. Alterations in p53 have been observed in over 50% of human tumors, including mutations in 28% of human breast tumors as well as functional inactivation of p53 through nuclear exclusion or binding to mdm-2 in an as yet unknown percentage of breast tumors. p53 is a cell cycle checkpoint protein that, in response to certain types of DNA damage, regulates progression through the cell cycle in concert with the cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} (17). In addition, p53 can regulate entry into the apoptotic process in concert with

apoptotic genes such as Bcl-2 (5). As recently reviewed, Wnt-1 is a major part of a signal transduction pathway involving β -catenin, the adenomatous polyposis coli (APC) tumor suppressor gene, and several down-stream target genes (18). In brief, in the absence of a Wnt-1 signal, β -catenin is phosphorylated by glycogen synthase kinase and associates with the APC protein. Formation of this complex leads to phosphorylation of APC, facilitating the binding of additional β -catenin, which is subsequently ubiquitinated and degraded by the proteasome complex. Activation of Wnt-1 leads to the inactivation of glycogen synthase kinase, causing β -catenin and APC to remain in a hypophosphorylated state. β -catenin does not bind to APC and instead accumulates in the cytoplasm where it binds to T-cell factor (Tcf)/ leukocyte enhancing factor (Lef). When β -catenin binds to Tcf/Lef, the complex translocates into the nucleus, apparently resulting in a constitutive complex that leads to transcriptional activation of genes involved in cell growth control. Although the APC / β -catenin interaction has been best studied in colon cancer, it has become clear that this pathway is important in breast carcinogenesis as well (18).

1.2 Purpose and Scope of the Work

The purpose of this DOD -funded study is to evaluate the effect of calorie restriction (CR; the most potent and broad acting dietary perturbation for inhibiting rodent tumor development) and the chemopreventive agents DHEA, genistein (in the form of a genistein-enriched soy extract) and 4-HPR, on spontaneous mammary tumorigenesis and relevant gene expression in *Wnt-1* TG mice with and without p53-deficiency. This will provide the initial step in characterizing these mice as models of spontaneous mammary tumorigenesis for cancer chemoprevention studies.

The specific aims of the proposal are:

1. To determine whether CR, DHEA, genistein or 4-HPR modulate the incidence, latency (time to palpable tumor) or burden (total number of mammary tumors and total tumor weight) of spontaneous mammary tumors in *Wnt-1* TG mice with or without wild-type p53 expression.

2. To characterize the mechanisms underlying interventions that inhibit mammary tumorigenesis by measuring in non-neoplastic mammary tissue collected 2 weeks after the onset of treatment (before tumor development) the expression (relative to controls) of the p53-dependent cell cycle regulator *Waf-1/p21* by Northern blot analysis as well as the p53-related apoptotic regulator *Bcl-2* and its negative inhibitor *Bax* by a competitive polymerase chain reaction (PCR) assay.
3. To evaluate the expression of RAR- β by in situ hybridization in mammary tumors (collected throughout the study) as well as in non-neoplastic mammary tissue collected after two weeks of treatment.
4. To evaluate the expression of *Brca-1* in mammary tumors (collected from the tumor study) and in non-neoplastic mammary tissue after 2 weeks of each treatment.

2.0 BODY

2.1 Original Statement of Work

Technical Objective 1: Treatment Effects on Spontaneous Mammary Tumorigenesis in *Wnt-1 TG* and p53-Deficient *Wnt-1 TG* Mice.

Task 1: Months 1-4. Mate wild-type females x *Wnt-1 TG* male mice and mate p53^{-/-} females by p53-deficient *Wnt-1 TG* males to produce 80 female wild-type, 80 female *Wnt-1 TG* and 80 female p53-deficient *Wnt-1 TG* mice for the study.

Task 2: Months 2-4. Genotype female offspring of above matings; randomize relevant genotypes into treatment arms.

Task 3: Months 2-15. Continue mice on diet treatments; monitor food consumption, body weights and mammary tumor development; kill mice when tumors are 1.5 cm in diameter; collect and store portions of mammary and other tissues in formalin for subsequent histopathologic analysis or flash freeze in liquid nitrogen and store at -80° C for subsequent molecular analyses.

Task 4: Months 16-19. Conduct histopathologic analysis.

Technical Objective 2: Treatment Effects on *Waf-1/p21*, *Bcl-2*, and *Bax* Expression in Non-Neoplastic Mammary Glands from *Wnt-1 TG* and p53-Deficient *Wnt-1 TG* Mice.

Task 5: Months 16. Randomize an additional 15 mice per genotype (3 mice/treatment group) to each of the 5 dietary treatments.

Task 6: Month 17. Collect mammary glands and other tissues after 2 weeks of treatment; store half in buffered formalin; flash freeze the remainder and store at -80° C

Task 7: Month 18. Isolate total RNA from frozen mammary tissues.

Task 8: Months 19-24. Analyze *Waf-1/p21* expression by Northern blotting and *Bcl-2* and *Bax* expression by competitive PCR analysis.

Technical Objective 3: Treatment Effects on RAR- β Expression in Mammary Tumors and Non-Neoplastic Mammary Glands.

Task 9: Months 2-15. Collection and storage of mammary tumors in buffered formalin.

Task 10: Month 18. Preparation of tissue sections for in situ hybridization.

Task 11: Months 19-24. Analysis of RAR- β expression by in situ hybridization.

Technical Objective 4: Treatment Effects on *Brca-1* Expression.

Task 12: Month 18. Isolate total RNA from frozen non-neoplastic mammary glands (collected and isolated as described in Tasks 6 and 7) and from frozen mammary tumors (collected as described in Task 3).

Task 13: Months 19-24. Analyze *Brca-1* expression by Northern blot analysis.

2.2 Progress report

2.2.1 Generation of Wild-Type, *Wnt-1* TG, and p53-Deficient *Wnt-1* TG Mice.

Our initial step was to develop a *Wnt-1* TG mouse colony at the M.D. Anderson Cancer Center to generate the needed mice for the study. Our funding began 10/1/96, and we had obtained 4 breeding pairs on 7/26/96 from Dr. Larry Donehower (Baylor College of Medicine), consisting of p53-/-: *Wnt-1* TG males x *Wnt-1* TG females, to begin the colony.

The development of this colony presented several challenges which we have solved through our breeding strategies. First, the p53-/- females have a high rate of dystochia, and thus experience difficulty with labor. There is also a significant gender skew in the p53-/- mice, with approximately a 4:1 ratio of males to females. In addition,

the Wnt-1 TG female does not lactate. We have thus established a colony consisting of ~50 male p53+/-:Wnt-1 TG mice and ~25 female p53+/- mice, and have generated 756 mice thus far to establish the colony and seed the tumorigenesis study. In an effort to enhance our numbers of female p53+/-:Wnt-1 TG mice, we also set-up (beginning at month 9 of the grant) 10 breeding pairs of p53+/- males x p53+/-:Wnt-1 TG females, and use 10 C3H mice to foster the pups. Our original estimates for colony development did not anticipate these challenges; thus our estimate of only 4 months to generate the mice drastically underestimated the amount of time required to develop the breeding colony and accomplish this task. All mice had been required to establish the colony until Month 10, when we had sufficient numbers to begin the tumor study.

2.2.2 Genotyping procedure for Wnt- and p53

Southern blotting had previously been used to genotype Wnt-1 TG and p53+/-:Wnt-1 TG mice, but we required a faster and less time-intensive method for the genotyping of the large numbers of mice we are generating. We therefore developed a PCR-based genotyping procedure for Wnt-1 which is used in tandem with our PCR-based protocol for p53 that we routinely use with our p53 colony. The protocol is as follows:

PCR PROTOCOL

(allele determination for WNT-1 transgenic mice)

Presence of a 350 bp product indicates the WNT-1 transgene is present. Absence of this band indicates the WNT-1 transgene is not present. A separate PCR (using different primers) should be performed on each sample to verify the integrity of the template/PCR process. Control DNAs supplied include W666 (p53 -/-; wnt-1 +), W672 (p53 +/-; wnt-1 -), W708 (p53 +/+; wnt-1 +), and W663 (p53 +/-; wnt-1 -).

PCR Primer Sets

Product size

WNT-1 (sense) 5'-GGA~~CT~~TGCTTCTCTTCATAGCC-3'
SV40 (antisense) 5'-CCACACAGGCATAGAGTGTCTGC-3'

350 bp

This PCR product runs closer to 400 bp rather than 350 bp

The SV40 primer is the "L" (late) or "plus" strand of SV40 at position 4198-4220. It serves as the "antisense" primer in this reaction because the transgene vector was constructed in this fashion.

Amplification Reagents

Rxn Buffer (10X)
dNTP mixture @ 2.5 mM each

2.5 ul
1.0 ul

5' primer (15 pmol)	
3' primer (15 pmol)	
Taq Polymerase (5U/ul, 1.5 U)	0.30 ul
H ₂ O to 25.0 ul	
DNA (Positive Control, ie. heterozyg. DNA)	>200.0 ng
DNA (Sample, ie. proteinase K prep)	1.0 ul
No DNA (ie. H ₂ O)	5.0 ul
Mineral Oil	2 drops

Amplification Cycle Conditions (Perkin Elmer PCR Kit)

Preheat thermocycler to 94°C

File 512- 31	94°C	2 min	1 Cycle
File 512- 32	94°C	30 sec	
	60°C	30 sec	
	72°C	1 min	40 Cycles
File 512- 10	Soak 4°C		

Add 5 ul of loading buffer (5X) to each reaction. Load 15ul (or more) per well on a 2% agarose gel.

2.2.3 Spontaneous Mammary Tumor Study

A randomized block design is being used, with the goal of 20 female mice/genotype/treatment, with three genotypes of interest: p53^{-/-} Wnt-1 TG, p53^{+/-} Wnt-1 TG and p53^{+/+} Wnt-1 TG. We also are monitoring tumor development in a group of 20 p53^{+/+} non-Wnt-1 TG mice (i.e, these mice are littermates of the p53-deficient Wnt-1 TG mice but are wild type for both p53 and Wnt-1). All mice in the study are housed 4/cage (except the calorie restricted mice, which are individually housed) in hanging polycarbonate cages, begin treatments by 6-8 weeks of age, are maintained on acidified tap water and are observed daily and their food consumption and body weights are measured once per week. All food is purchased from Research Diets, Inc., (New Brunswick, NJ). Once a palpable tumor is detected, the growth of that tumor is monitored using calipers. Once a mouse has a 1.5 cm diameter tumor, that mouse is euthanized and its mammary glands and other tissues are collected and stored for subsequent whole mount, molecular or histopathologic analyses. In addition, blood is collected from each mouse immediately after euthanization for plasma analyses. All methods have been reviewed

and approved by the MDACC Institutional Animal Care and Use Committee.

2.2.3.1 p53^{-/-} Wnt-1 TG Mice

We have continued to have great difficulty in generating sufficient numbers of female p53^{-/-} Wnt-1 TG mice, and thus to potentially increase our sample size for this genotype we randomized male and female p53^{-/-} Wnt-1 TG mice into two separate groups. The male p53^{-/-} Wnt-1 TG mice develop spontaneous mammary tumors similarly to the females; in our hands, male p53^{-/-} Wnt-1 TG mice averaged 10.4 weeks on study compared with 9.2 weeks on study for female p53^{-/-} Wnt-1 TG mice. We have completed our chemoprevention studies in male (total of 29) and female (total of 26) p53^{-/-} Wnt-1 TG mice. As shown in table 1, the p53^{-/-} Wnt-1 TG mice were randomized into one of three groups: control (AIN-76A diet only); compound 8354 (AIN-76A diet containing 0.2% fluasterone, aka, compound 8354) or 4-HPR (AIN-76A diet containing 4 ppm 4-HPR. The compound 8354 was substituted for DHEA (which was originally proposed) since it is broadly recognized as a more promising human chemopreventive agent than the parent DHEA; the fluasterone is more potent, has fewer adverse side effects (particularly weight loss), and is much less androgenic and estrogenic than DHEA.

As shown in Table 1, relative to control male p53^{-/-} Wnt-1 TG mice (mean time to death [MTD] =10.4 weeks), male mice receiving compound 8354 (13 weeks; p=0.15) and 4-HPR (11.4 p=0.30) demonstrated non significant delays in spontaneous tumor development. All mice spontaneously developed mammary adenocarcinomas and were euthanized when those tumors reached 1.5 cm. . Similarly, relative to control female p53^{-/-} Wnt-1 TG mice (MTD=9.2 weeks), compound 8354 (MTD=11.1 weeks, p=0.17) and compound 8354 (MTD=10.4 weeks, p=0.18) exerted non-significant delays in spontaneous tumor development. These findings are also illustrated in Figs. 1 and 2 in the form of Kaplan-Meier survival curves.

Since male and female p53^{-/-} mice developed sponaneous tumors at a similar rate, we pooled data from the two genders to increase statistical power; relative to control p53^{-/-} Wnt-1 TG mice (both genders combined, MTD=9.7, n=18), 8354-treated mice (MTD=12.2, n=17, p=0.051) and 4-HPR-treated mice (MTD= 11.1, n=20, p=0.11)

demonstrated a borderline statistically-significant delay in mammary tumor development and death.

2.2.3.1 Female p53+/- Wnt-1 TG Mice

Female p53+/- Wnt-1 TG mice were much easier to generate than the p53-/- mice, so we are to date successful in completing an analysis of 4 separate preventive interventions in these mice. One hundred and four female p53+/- Wnt-1 TG mice were randomized to receive 1) control diet (same as above); 2) compound 8354 (same as above); 3) 4-HPR (same as above); 4) soy diet (AIN-76A diet with 7.5% of a phytochemical enriched soy extract from Arthur Daniels-Midland); 5) calorie restriction (60% of control carbohydrate calorie consumption and 100% of all other nutrients). The soy extract was used instead of purified genistein (as originally proposed) since inconsistent results have been reported with the purified isoflavone. The soy extract contains high levels of genistein as well as other isoflavones such as daidzein which could contribute to the chemopreventive activity.

As shown in table 1, relative to control female p53+/- Wnt-1 TG mice (MTD =15.7 weeks, n=21), mice receiving compound 8354 (MTD=23.1 weeks; n=21; p=0.006), 4-HPR (MTD=23.1; n=19; p=0.014) and soy (MTD=21.2; n=20; p=0.04) demonstrated modest but statistically significant delays in spontaneous tumor development and death. With the exception of two 4-HPR-treated mice and two soy-treated mice, which remain alive and tumor-free thus far, all mice spontaneously developed mammary adenocarcinomas and were euthanized when those tumors reached 1.5 cm. Remarkably, there are still 16/23 calorie restricted p53+/- Wnt-1 TG mice still alive through more than 35 weeks of study. As illustrated in Figure 3 (Kaplan-Meier Survival curves), compound 8354, 4-HPR and soy all exert moderate delaying effects against spontaneous mammary tumor development in p53+/- Wnt-1 TG mice, while calorie restriction exerts highly potent (~300-400%) delaying effects in these mice

The p53+/+ Wnt-1 TG mice demonstrate delayed mammary tumor development relative to the p53-/- or p53+/- Wnt-1 TG mice, and thus are still largely under study. Table 1 and Figure 4 illustrate a similar trend (observed in the p53-/- and p53+/- Wnt-1 TG mice) of a protective effect of calorie restriction, soy and 4-HPR in these mice. Interestingly, the protective effect of 8354 observed in the p53+/- Wnt-1 TG mice and, to a lesser extent, in the p53-/- Wnt-1 TG mice,

is not evident in the p53+/+ Wnt-1 TG mice. The MTD for the controls is 27.9 weeks compared to 26.8 weeks for the 8354-treated mice; there are still a small number of mice alive and tumor free in each group. Over half of the 4-HPR and soy-treated p53+/+ Wnt-1 TG mice are also still alive, and nearly all of the calorie restricted mice remain alive and tumor free, suggesting these mice will also demonstrate a delay in tumor development in response to 4-HPR, soy and CR.

The protective effects of the different diets appear to be primarily due to an effect on time to tumor development; with the exception of the calorie restricted groups, which still have 70-80% of their mice alive at this point, no differences were observed in incidence of mammary tumors, multiplicity, or in the time from palpable tumor to tumor death (just over two weeks for all groups; data not shown). Our previous experience with calorie restriction suggests that those mice will also develop mammary tumors, albeit much later than the controls. It will be interesting to see if calorie restriction also delays the progression from a palpable tumor to a 1.5 cm tumor, our criterion for euthanasia.

The final genotype being assessed involves a group of 20 p53+/+ non-Wnt-1 TG mice which have been included as negative controls for this study. These mice began nearly 6 months earlier than most of the other mice on study; 19/20 remain alive and tumor-free through 64 weeks. The one mouse that died had a lymphoma; no mammary tumors have been observed in this group.

We have also completed the in-life portion of the short-term study proposed to complete aims 2, 3 and 4 and are now in the final phases of analyses with these tissues, as well as tissues from the main tumor study. In collaboration with Dr. Barbara Davis, a Veterinary Pathologist at the NIEHS and expert in BRCA-1 and BRCA-2 analysis by in situ hybridization, we have completed the histopathologic assessment of those mice that have died up to Dec. 1, 1998 and have begun to assess BRCA-1 and BRCA-2, PCNA and APOTAG expression in those tumors (Aim 4). RNA has been sent to Dr. Tom Wang (NCI) for the Bcl-2/Bax analysis (Aim 2) and unstained sections on selected samples are being cut for RAR- β analysis in collaboration with Dr. Reuben Lotan. We have also established a collaboration with Dr. Henry Thompson of the AMC Cancer Center in Denver, CO to conduct whole mount analysis to determine the combined gene-chemopreventive effects on mammary gland development and

morphology. Figures 5 a-e show representative images of our whole mounts; we await sufficient tissue samples from our soy and calorie restricted groups of p53^{+/-} Wnt-1 TG mice and from our p53^{+/+} TG mice to fully assess the effects of genotype and treatment, although p53 deficiency is clearly influencing the extent of ductal branching and extension in the gland. Funding for the completion of these studies is being covered by Departmental funds.

Summary and Conclusion

Due to the nature of this study, with much of the first year occupied by generating the mice for the spontaneous tumorigenesis study, we can primarily report on our progress towards characterizing the response of Wnt-1 TG mice (with and without p53-deficiency) as a model for chemoprevention studies, our primary aim. We expanded the scope of the study to include male p53^{-/-} Wnt-1 TG mice and female p53^{+/-} Wnt-1 TG mice along with the female p53^{-/-} and p53^{+/+} Wnt-1 TG originally proposed. This turned out to be an excellent decision, since the p53^{-/-} are so difficult to generate. In addition, spontaneous mammary tumor development in p53^{-/-} Wnt-1 TG appears to be somewhat too aggressive for utility as a chemopreventive model, as tumors appeared within just a few weeks after initiating treatments. Also, no gender difference was observed in the mean time to tumor development in the p53^{-/-} Wnt-1 TG mice; this made it possible to pool data from the two genders to increase our power, but it also suggests that the mechanism underlying spontaneous tumor development in mice overexpressing Wnt-1 and with p53 completely inactivated may not be particularly relevant to human breast carcinogenesis, where male breast cancer is rare.

At the other extreme, the p53^{+/+} Wnt-1 TG mice appear to be too slow in developing mammary tumors at high rates; many of those mice appear to remain tumor free for 1 year or even longer. Also, the distribution of the mean times to death among the p53^{+/+} Wnt-1 TG mice is very broad; some mice in this group developed tumors in the first few months of the study while many remain tumor free for over a year. This variability can make the interpretation of chemopreventive responses very difficult. In contrast, the p53^{+/-} Wnt-1 TG mice appear to be very well suited as a spontaneous mammary tumor model for diet and chemoprevention studies. The mean time to death is about 16 weeks (i.e., about 24 weeks old), with

a fairly tight distribution around that mean (\pm ~8 weeks). Moderate but statistically significant responses to the chemopreventives 8354, 4-HPR and a high soy diet were observed in these mice, and an incredibly strong delaying response was seen with calorie restriction in these mice, suggesting that these mice provide a relevant and sensitive model for chemopreventive studies.

We plan to capitalize on the tissues from the chemopreventive study as well as those collected from a short-term treatment to complete the proposed biomarker analyses over the next 2-3 months. This will provide important information about the mechanisms underlying efficacious preventive interventions. The chemopreventive study data in p53^{-/-} Wnt-1 TG mice has already been presented at the Barton Creek Conference "Genetic susceptibility to cancer" in Austin, TX on December 4, 1998, and an abstract of the full chemopreventive study has been accepted for oral presentation at the 1999 American Society for Preventive Oncology Annual meeting. In fact, this abstract received the second-highest priority score of all the abstracts submitted to the meeting and was accepted for oral presentation in the Plenary Session (the top 6 abstracts) of the meeting. Based on this abstract, a manuscript has been invited for submission to Cancer Epidemiology, Biomarkers and Prevention for February, 1999, and we anticipate a second manuscript on the calorie restriction effect in the early spring. We also anticipate that the marker data will generate important manuscripts. Thus, despite some delay in generating the needed mice for the study, the project has been highly successful, and has resulted in the characterization of an important new spontaneous mammary tumor model for cancer chemoprevention research.

Personnel Supported By the Grant

All salary support went to cover the salary of Jiancheng Shen, a Post-doctoral Fellow in Dr. Hursting's laboratory who was responsible for conducting the technical aspects of the study.

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Table 1. Mean Weeks on Study By Treatment and Genotype

Gender/ Genotype	Control	8354	4-HPR	Soy	CR
MKO Wnt	10.4 ± 3.4 (n=7)	13.0 ± 5.9 (n=10) p v. control= 0.15	11.4 ± 4.2 (n=12) p v. control= 0.30	-	-
FKO Wnt	9.2 ± 2.9 (n=11)	11.1 ± 5.5 (n=7) p v. control= 0.17	10.4 ± 2.3 (n=8) p v. control= 0.18	-	-
KO Wnt (Genders combined)	9.7 ± 3.1 (n=18)	12.2 ± 5.7 (n=17) p v. control= 0.05	11.1 ± 3.6 (n=20) p v. control= 0.11	-	-
FHET Wnt	15.7 ± 8.2 (n=21)	23.1 ± 9.9* (n=21) p v. control= 0.006	23.1 ± 12.1* (n=19; 2 alive) p v. control= 0.014	21.2 ± 11* (n=20; 2 still alive) p v. control= 0.04	>40** (16/23 still alive through ~35 weeks) p v. control< 0.001
FWT Wnt	27.9 ± 16.9 (n=13; 3 still alive)	26.8 ± 11.4 (n=20; 1 still alive) p v. control= 0.41	10/21 still alive	16/20 still alive	18/20 still alive

*different from control (for that gender/genotype) at p<0.05

** different from control (for that gender/genotype) at p<0.001

MKO Wnt = Male p53^{-/-} Wnt-1 transgenic

FKO Wnt = Female p53^{-/-} Wnt-1 transgenic

FHET Wnt = Female p53^{+/-} Wnt-1 transgenic

FWT Wnt = Female p53^{+/+} Wnt-1 transgenic

Control = AIN-76A diet

8354 = AIN-76A diet + 0.2% fluasterone (compound 8354)

HPR = AIN-76A diet + 4ppm fenretinide (4-HPR)

Soy = AIN-76A diet + 0.45% phytochemical-enriched soy extract

CR = modified AIN-76A providing 60% of carbohydrate calories; 100% of all other nutrients (40% calorie restriction)

Note: 19/20 FWT non-transgenic mice (wild-type for both p53 and Wnt) maintained on control diet are still alive and tumor free through 64 weeks of study

Figure 1

**Effects of Fluasterone or 4-HPR on Survival in
Female p53^{-/-} Wnt-1 Transgenic Mice**

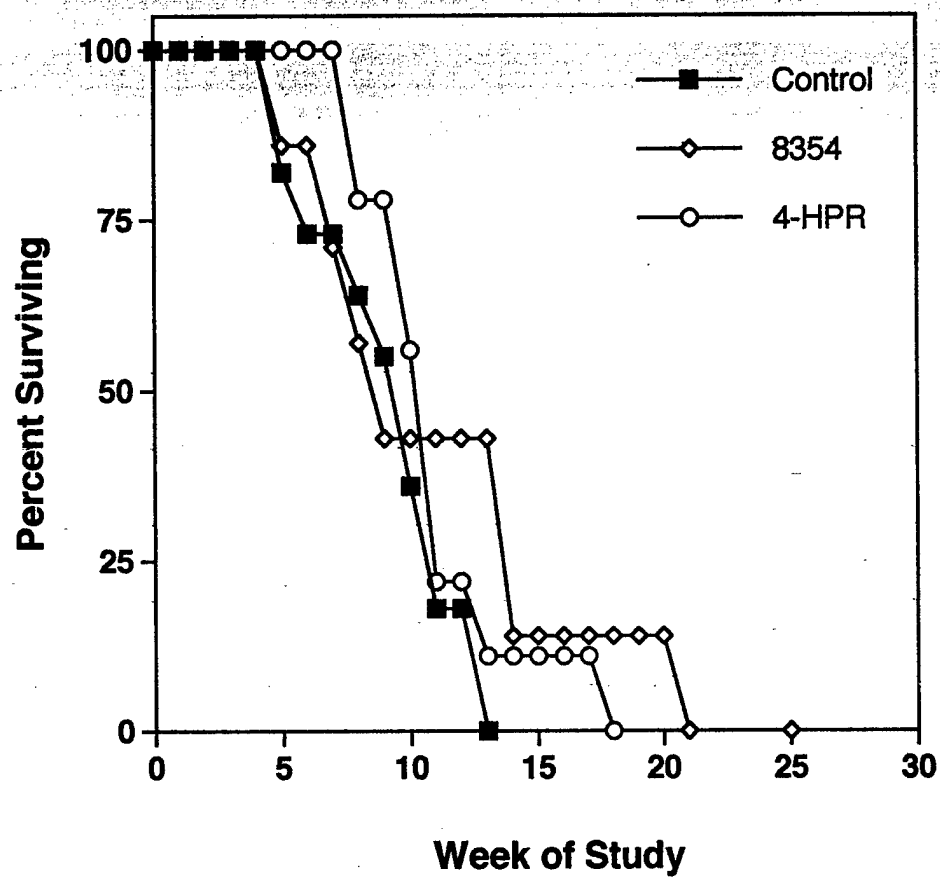


Figure 2

**Effect of Fluasterone and 4-HPR on Survival
in Male p53^{-/-} Wnt-1 Transgenic Mice**

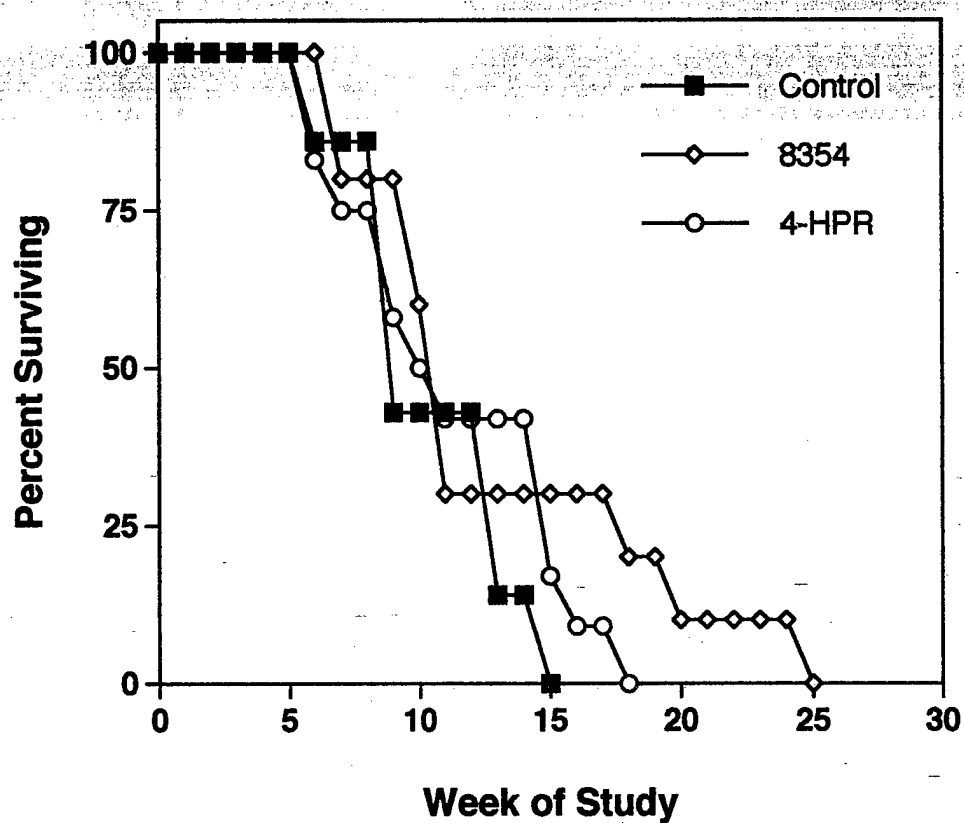


Figure 3

**The Effect of Treatment on Survival
in Female p53^{+/-} Wnt-1 Transgenic Mice**

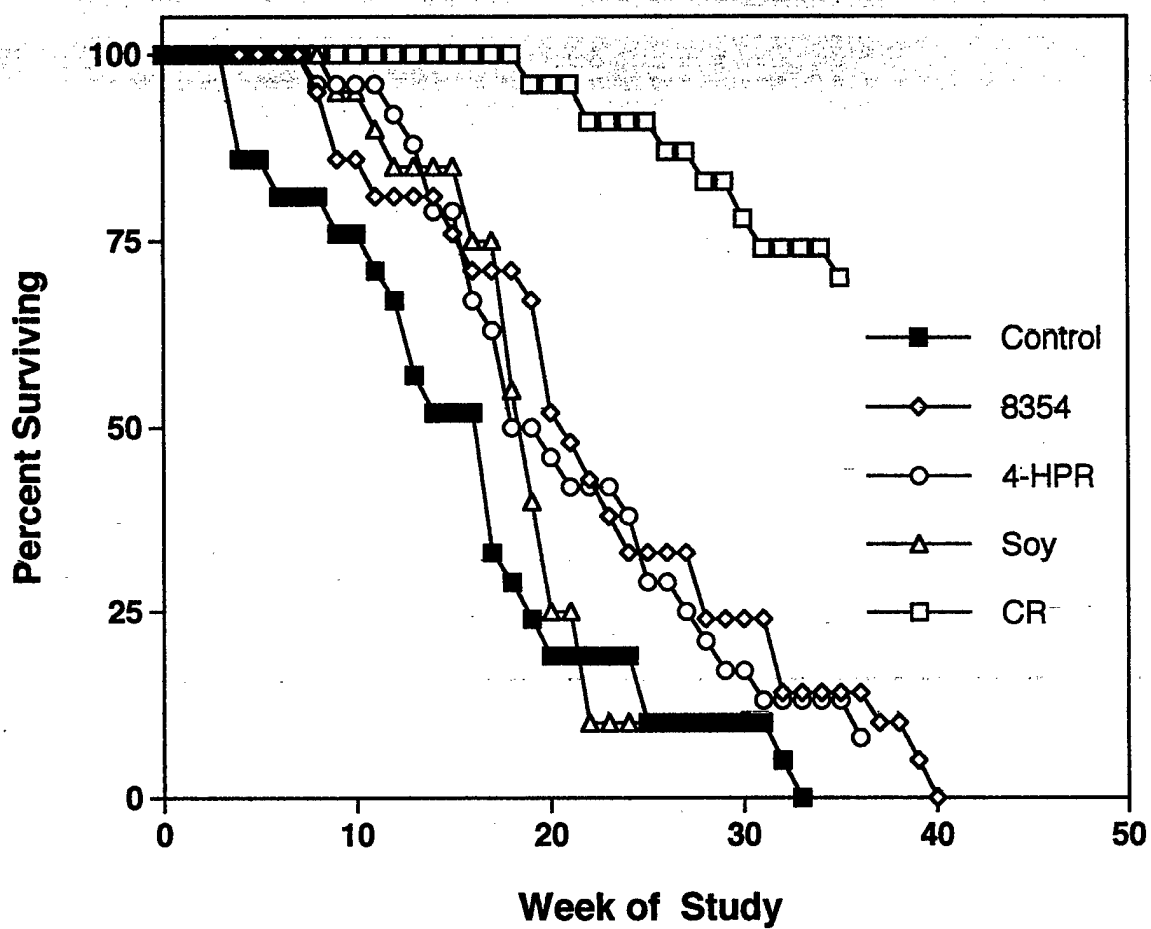
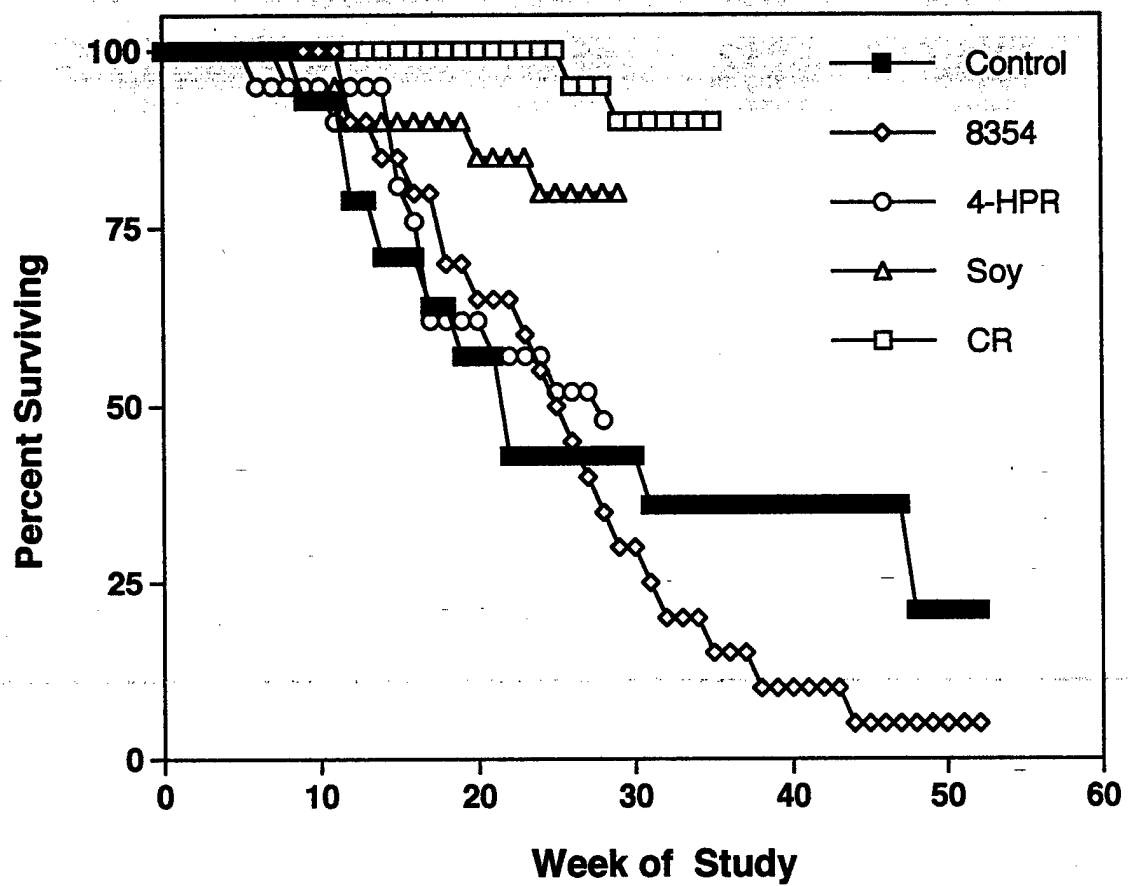


Figure 4

**Effect of Treatment on Survival in
Female p53^{+/+} Wnt-1 Transgenic Mice**



Examples of whole mounts

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8300

wnt-1/p53 control

wnt-1/p53 8354

wnt-1/p53 4HPR

Mammary whole
mount examples of
wnt-1/p53

Tumor examples of
wnt-1/p53

5a

Mouse #109

p53-/- Wnt-1 TG

Control

Inguinal Mammary Gland



wnt-1/p53 control

wnt-1/p53 8354

wnt-1/p53 4HPR

Mammary whole
mount examples of

wnt-1/p53

Tumor examples of
wnt-1/p53

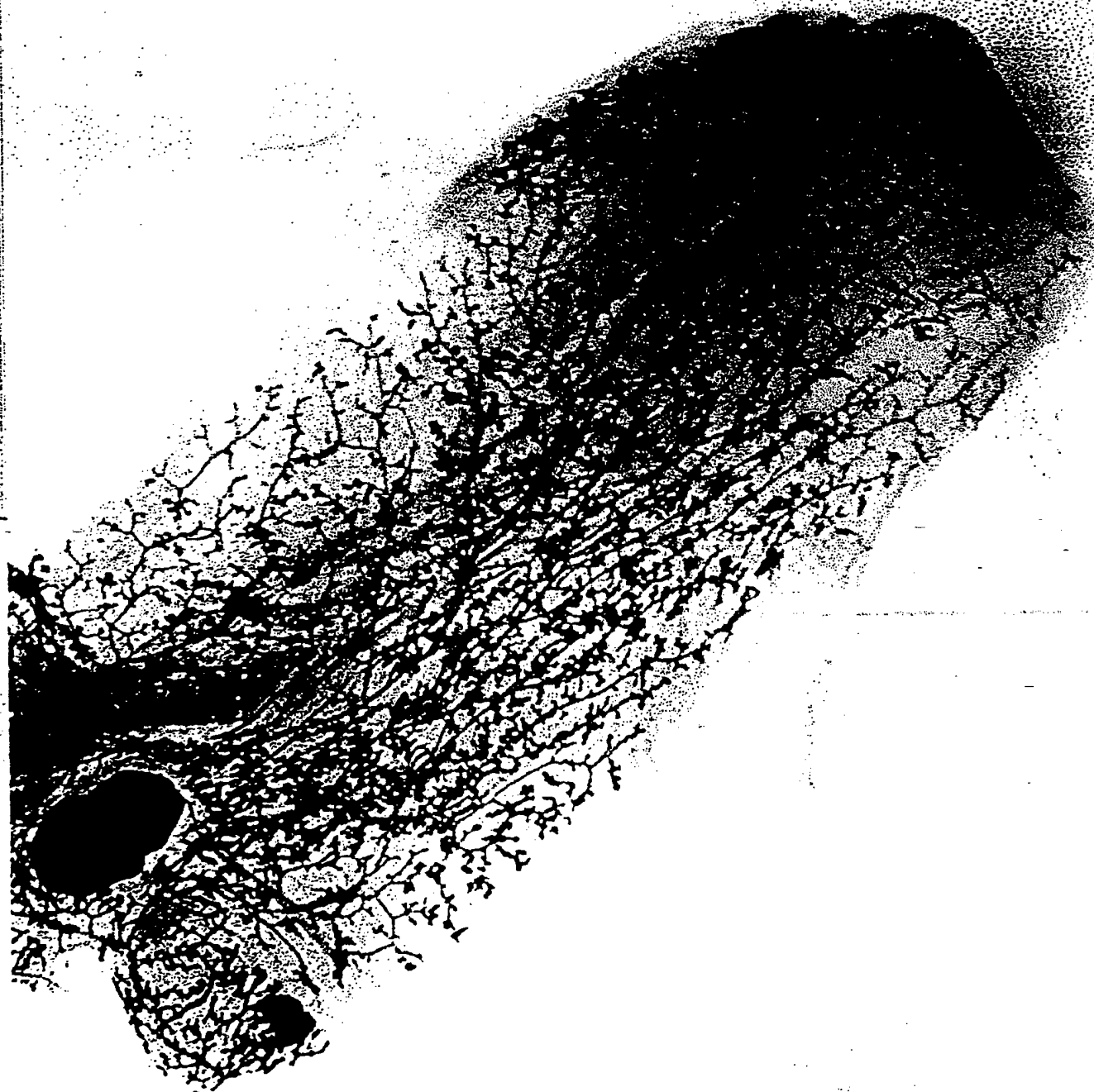
sb

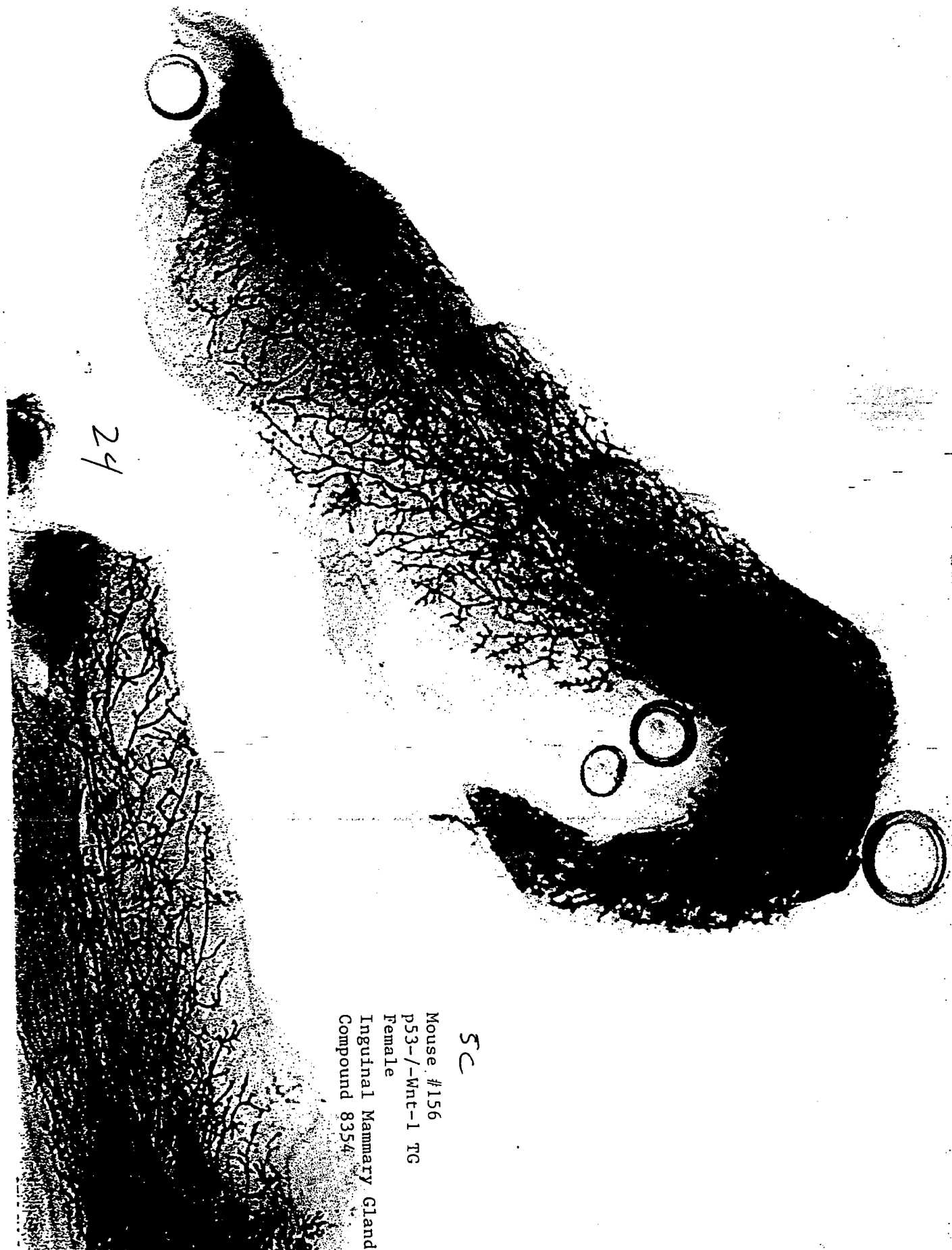
Mouse # 210

p53+/- Wnt-1 TG

Inguinal Mammary Gland

Control





5C

Mouse #156
p53-/-Wnt-1 TG
Female
Inguinal Mammary Gland
Compound 8354

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SECRET

wnt-1/p53 control

wnt-1/p53 8354

wnt-1/p53 4HPR

Mammary whole
mount examples of
wnt-1/p53

Tumor examples of
wnt-1/p53

SD

Mouse #760
p53+/- Wnt-1
Female
Inguinal Mammary Gland
Compound 8354

Se

Mouse #361

p53+/+ Wnt-1 TG Female

Compound 8354

Inguinal Mammary Gland

26



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NEW

wnt-1/p53 control

wnt-1/p53 8354

wnt-1/p53 4HPR

Mammary whole
mount examples of
wnt-1/p53

Tumor examples of
wnt-1/p53

Control
Inguinal Gland
Mouse #760
p53+/- Wnt-1 TG

AMERICAN SOCIETY OF PREVENTIVE ONCOLOGY - ABSTRACT FORM

For Submission of Presented Papers at the 23rd Annual Meeting in Houston, Texas, March 14-16, 1999.

An abstract, consisting of 150-200 words, **MUST BE TYPED** in the box below. **Title** should be brief and clearly state content of paper. Please list **last names and first initials** of co-authors and **institution** of 1st author. The body of the abstract should be organized as follows: 1. Purpose of study (one sentence if possible). 2. Simple statement of methods. 3. Summary of results (adequate to support conclusion). 4. Statement of conclusions. (Do not use phrases such as "The results will be discussed.") 5. Authors must use 12-point type, and no illustrations are permitted within abstracts. Space has been allocated to publish the top-ranked abstracts in the March '99 issue of Cancer Epidemiology, Biomarkers and Prevention. Do not reduce the abstract to "make it fit", as it will be reduced for reproduction in the journal. Do not type across the "borders" of the abstract box or the line(s) will show up in the program.

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A Novel Mammary Tumor Model For Cancer Prevention Studies.
Hursting S, Baum L, Shen J, Thompson H, Davis B, Clinton S
U.T.- M.D. Anderson Cancer Center, Houston, TX 77030.

Like many kindreds diagnosed with the Li-Fraumeni familial cancer syndrome, heterozygous p53-deficient (p53+/-) mice have only one functional allele of the p53 tumor suppressor and are prone to a variety of neoplasms at mid-life; the median time to tumor-related death (MTD) of p53+/- mice is 18 months. We have previously reported that spontaneous tumorigenesis in p53+/- mice can be suppressed by several preventive interventions. Since mammary tumors in these mice are rare, we have crossed p53+/- mice with *Wnt-1* transgenic (TG) mice to develop a rapid spontaneous mammary tumor model. *Wnt-1* TG mice develop mammary tumors at a high rate by 1 year of age due to mammary gland overexpression of the *Wnt-1* oncogene. We have shown that p53-deficiency accelerates mammary tumorigenesis in these mice with 100% of p53-/- *Wnt-1* TG mice and p53+/-*Wnt-1* TG mice dead from mammary tumors by 5 months and 8 months of age respectively. To test their response to cancer preventive regimens we randomized 100 female p53+/- *Wnt-1* TG mice (5 weeks of age, 20 mice/treatment) to receive: 1) control diet (AIN-76A diet); 2) fenretinide (AIN 76A diet with 0.04% w/w fenretinide); 3) fluasterone (AIN-76A diet with 0.2% fluasterone); 4) soy (AIN 76A diet with 0.45% phytochemical-enriched soy extract); or 5) calorie restriction regimen (40% reduction in carbohydrate calorie intake relative to the control group). All mice were euthanized once a tumor reached 1.5 cm in diameter. We found that, relative to the control group (MTD=22 weeks), the fenretinide (MTD=32 weeks, p=0.03) and fluasterone (MTD=29 weeks, p=0.04) groups displayed moderate but significant delays in tumor development and death, while the soy (MTD > 20 weeks) and calorie restricted (MTD > 40 weeks) groups experienced highly significant delays in spontaneous mammary tumor development. Mechanistic studies are underway to determine the cellular and molecular response underlying the preventive effects of these interventions. We conclude that p53+/- *Wnt-1* TG mice provide a rapid and sensitive model of spontaneous mammary tumorigenesis for characterizing breast cancer prevention strategies. (Funded by DAMD 17-96-6216 and P30 ES07784).

TYPE name, address, and telephone number of AUTHOR WHO WILL BE PRESENTING the work at ASPO:

NAME Stephen D. Hursting, Ph.D., M.P.H.

INSTITUTION University of Texas-M.D. Anderson Cancer Center DEPARTMENT Epidemiology

ADDRESS 1515 Holcombe Blvd., Box 189

CITY, STATE, ZIP Houston, TX 77030

TELEPHONE (713) 745-2492

FAX (713) 792-0807